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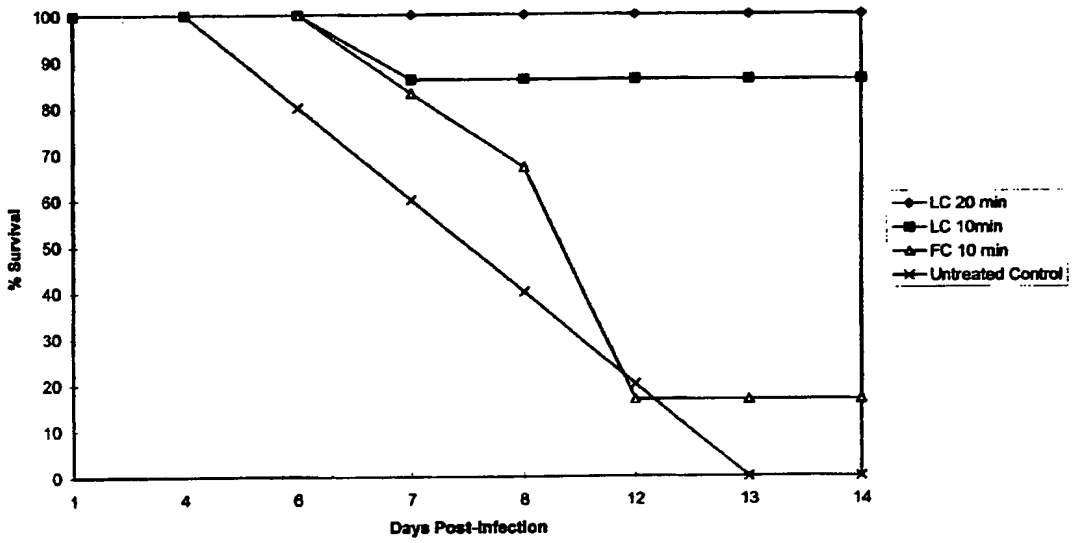
(54) **ADMINISTRATION, GRACE A UN AEROSOL, DE FLUOROQUINOLONE ENCAPSULEE DANS DES LIPOSOMES**

(54) **AEROSOL DELIVERY OF LIPOSOME-ENCAPSULATED FLUOROQUINOLONE**



(57) L'efficacité thérapeutique de la fluoroquinolone encapsulée dans des liposomes, pour le traitement des infections respiratoires, est améliorée grâce à une technique d'administration permettant de diriger le médicament directement vers le site d'infection à l'aide d'un aérosol.

(57) The therapeutic efficacy of liposome-encapsulated fluoroquinolone for the treatment respiratory infections is enhanced by providing a delivery system capable of targeting the drug directly to the infection site using aerosol delivery.

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AEROSOL DELIVERY OF LIPOSOME-ENCAPSULATED FLUOROQUINOLONE

ABSTRACT

The therapeutic efficacy of liposome-encapsulated fluoroquinolone for the treatment respiratory infections is enhanced by providing a delivery system capable of targeting the drug directly to the infection site using aerosol delivery.

AEROSOL DELIVERY OF LIPOSOME-ENCAPSULATED FLUOROQUINOLONE

FIELD OF THE INVENTION

The present invention pertains to a method for the treatment and prevention of respiratory infections using therapeutic aerosols containing liposome-encapsulated fluoroquinolone. This method delivers concentrated doses of liposome-encapsulated fluoroquinolone directly to the site of infection in the body, thereby enhancing its therapeutic efficacy.

BACKGROUND OF THE INVENTION

The fluoroquinolones as a class are potent, broad-spectrum antibacterial agents that are effective against a number of gram-negative and gram-positive microorganisms. They block bacterial deoxyribonucleic acid (DNA) replication by inhibiting DNA gyrase which is an essential enzyme that catalyzes the bacterial DNA replication system. For example, ciprofloxacin has shown to demonstrate good *in vitro* bactericidal activity against a number of pathogens that causes respiratory infections including *Mycobacterium tuberculosis* (Antimicrob. Agents Chemother., 1984, 26: 94-96; Tubercle, 1987, 68: 267-276), *Mycobacterium avium-intracellulare* and *Haemophilus influenzae* (Antimicrob. Agents Chemother., 1986, 29: 386-388), *Pseudomonas aeroginosa* (Infection, 1983, 11: 326-328) and *Neisseria meningtidis* (Antimicrob. Agent Chemother., 1984, 25: 319-326). Despite promising *in vitro* data, the clinical use of oral or intravenous ciprofloxacin in humans for fighting respiratory infections has not gain widespread acceptance. This may be due in part to the relative unfavorable

pharmacokinetic profiles of ciprofloxacin in the lower respiratory tract which includes relatively short elimination time, $t_{1/2}$ of 1.0 to 1.6 hour, and low AUC[1] of 43 to 113 mg.h/L (Quinolones Bulletin, 1993, 10: 1-18).

Recently, applicant provided a method for improving the therapeutic efficacy of ciprofloxacin by encapsulating ciprofloxacin within liposomes (Canadian Patent Application No. 2,101,241). When liposome-encapsulated ciprofloxacin was administered to mice intranasally, it was found that the retention of the drug in the lungs was enhanced significantly with $t_{1/2}$ from 1-2 to 8-10 hours. Moreover, the treatment for the pathogen, *Francisella tularensis*, was enhanced several-fold by using liposomal ciprofloxacin. However, it is believed that the therapeutic efficacy of liposome-encapsulated ciprofloxacin against respiratory infections can be further enhanced by providing a drug delivery system capable of depositing the drug directly to the infection site.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a liposomal fluoroquinolone aerosol drug delivery system which is capable of delivering the drug directly to an infection site.

In accordance with one aspect of the present invention, there is provided an aerosol composition comprising a therapeutically effective amount of liposome-encapsulated fluoroquinolone. Preferably, the fluoroquinolone is selected from the group consisting of amifloxacin (AMI), cinoxacin (CIN), ciprofloxacin (CIP), danofloxacin (DAN), difloxacin (DIF), enoxacin (ENO), enrofloxacin (ENR), fleroxacin (FLE), irloxacin (IRL), lomefloxacin (LOM),

[1] "AUC" stands for the area under the curve, and is used to determine the bioavailability of drugs. The higher the area under the curve, the better will the drug be for therapeutic application.

miloxacin (MIL), norfloxacin (NOR), ofloxacin (OFL), pefloxacin (PEF), rosoxacin (ROS), rufloxacin (RUF), sarafloxacin (SAR), sparfloxacin (SPA), temafloxacin (TEM) and tosufloxacin (TOS).

The aerosol composition is useful for prevention and treatment of respiratory infections caused by, for example, *Francisella tularensis*. The aerosolized liposome-encapsulated fluoroquinolone can be in the form of a liquid or dry powder.

In an embodiment of the present invention, the amount of liposome-encapsulated ciprofloxacin in aerosol form which is effective in treating infection by *F. tularensis* is approximately in the range of 5 μg/mL to 40 μg/mL. Preferably, at least 50% of the liposomal ciprofloxacin are in the form of particles having a diameter of 0.5 to 5.0 μm, and preferably a diameter of 3.45 μm. The particles further have a peak particle count (10^6) in the range of about 1.2 to 4.4, and preferably of 4.35.

In accordance with another aspect of the invention, there is provided a method for administering liposome-encapsulated fluoroquinolone in aerosol form using a jet nebulizer, such as the nebulizer PurRD Raindrop from Puritan-Bennett of Lenexa, KS or a metered dose inhaler.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing results of tests relating to the therapeutic efficacy of liposome-encapsulated ciprofloxacin aerosols versus that of free liposome-encapsulated ciprofloxacin aerosols in the prevention of respiratory infections.

Figure 2 is a graph showing results of tests relating to the therapeutic efficacy of liposome-encapsulated ciprofloxacin aerosols versus that of unencapsulated ciprofloxacin aerosols in the treatment of respiratory infections.

DETAILED DESCRIPTION

As used herein, the terms "APS" means the Model #3320 aerodynamic particle sizer, from TSI Inc., St. Paul, MN.; "GSD" means geometric standard deviations; "MMAD" means mass mean aerodynamic diameter, and is the aerodynamic diameter above which 50% of the total particle mass resides; "PBS" means phosphate buffered saline; "PPC" means peak particle counts; PP_i means inorganic phosphate group; and REV means reverse phase evaporation vesicles.

In the treatment of disease, aerosol administration provides a valuable method by which a drug may be delivered. This method is particularly efficient in the treatment of diseases involving airway obstruction, such as asthma, bronchitis, and emphysema. Aerosol therapies may also be used for mucolytics which decrease the thickness or viscosity of mucus in diseases involving abnormal mucus secretion, such as pneumonia, bronchitis, and cystic fibrosis, and antibiotics (in the treatment of lung infections). Furthermore, aerosols are utilized for clinical investigation and diagnosis, for example, for the delineation of airway reactivity using bronchoconstrictors.

One widely used method of generating aerosol particles involves the use of a jet nebulizer. A jet nebulizer operates on compressed air to propel a liquid drug formulation into aerosol particles. The output of aerosol droplets differs from one jet nebulizer to another. A

nebulizer can often handle a wide range of air pressures, and changes in air pressure can vary the aerosol output and particle sizes enormously. The composition of the liquid formulation can also influence the aerosol output.

The droplet size of the aerosol generated is influenced by much the same factors as the aerosol output. An increased in the air pressure used to operate the jet nebulizer will decrease particle size. Particle size is one of the main factors which govern the successful passage of a drug from the outlet of a nebulizer to an infection site. For an aerosol delivery system to be effective in treating pulmonary pathogens, the particle size should generally not exceed about five microns. Another important factor governing the efficacy of an aerosol delivery system is the quantity of aerosol that will be deposited on the target cell or tissue. This quantity is usually express in peak particle count (PPC). The efficiency of an aerosol delivery system is directly proportional to the PPC which it exhibits.

Chemicals

The phosphatidylcholine, phosphatidylserine, and cholesterol used for the preparation of liposomes were purchases from Avanti Polar Lipids (Alabaster, AL.). Ciprofloxacin (Bayer Corp. of Canada, Etobicoke, Ontario) was purchased through a local pharmacy.

Aerosol nebulizers

Table 1 identifies the supplier for each commercially available jet nebulizer used in this study.

Table 1: Jet Nebulizers and Their Respective Supplier

Jet Nebulizers	Suppliers	Location of Suppliers
1. A1800	ARS Vital Aire	Edmonton, Alberta, Canada
2. DVB7427	Devilbiss	Somerset, PA.
3. DVB5601	Devilbiss	Somerset, PA.
4. Microcirrus	DHD Medical Products	Canastota, NY.
5. Hosp 3753	Hospitak	Lisdenhurst, NY.
6. Hosp 952	Hospitak	Lisdenhurst, NY.
7. HudTU	Hudson RCI	Tumecula, CA.
8. HudUD2	Hudson RCI	Tumecula, CA.
9. HudMM	Hudson RCI	Tumecula, CA.
10. Int 111222OE	Intertech.	Bannockburn, IL.
11. Marq	Maruest Medical Products Inc.	Englewood, CO.
12. PurRD Raindrop	Puritan-Bennett	Lenexa, KS.

Animals

Six-week old BALB/c female mice were obtained from the mouse breeding colony at Defense Research Establishment Suffield (DRES) in Alberta, Canada, with breeding pairs purchased from Charles River Canada LTD. (St. Constant, Quebec, Canada). The use of animals described in this study was approved by the DRES Animal Care Committee. Care and handling of animals described in this study followed guidelines set out by the Canadian Council on Animal Care.

Bacteria

Francisella tularensis Live Vaccine Strain (LVS, ATCC 296684, American Type Culture Collection, Rockville, Md.) was cultured on the cysteine heart agar plates supplemented with 5% defibrinated rabbit blood (Remel Labs, Lenexa, Kans.) for four days in 5% CO_2 as described in the following reference: J. Infec. Dis., 1993, 168:793-794. Colonies were then selected for growing in modified Mueller-Hilton broth (Difco Laboratories) supplemented with ferric PP_i and IsoVitaleX (Becton Dickinson, Cackeysville, Md.). The broth cultures were incubated at 37°C for 4-5 days. The cultures were then aliquoted and frozen in 10% dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO.). For determining the 50% lethal dose (LD_{50}), aliquots were thawed and diluted serially in sterile PBS prior to administration into animals.

Preparation of liposome-encapsulated ciprofloxacin

The liposomes used for the encapsulation of ciprofloxacin were prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos (see Proc. Natl. Acad. Science, 1978,

75: 4194-4198) and by the remote-loading procedure using ammonium sulfate gradient described in Antimicrob. Agents Chemother., 1995, 39: 2104-2111. The liposomes were made from egg phosphatidylcholine and cholesterol in a molar ratio of 1:1, and the content of ciprofloxacin concentration used was 30 mg/mL.

Generation and characterization of liposome aerosols

A volume of 3 mL of liposome-encapsulated ciprofloxacin was added to each jet nebulizer reservoir. Aerosols were generated by the nebulizers using dry compressed air at 40 PSI with flow rates of 4 or 6 L/min until the reservoir was dry (between 10 to 20 minutes). Aerosol particles were analyzed using the APS and the APS Advanced Software, version 2.9 purchased from TSI Inc. Aerosol analysis was initiated after 2 minutes of equilibration and was carried out continuously for every 30 seconds until the end of each run. The aerosols particles generated by each nebulizer were characterized for their MMAD, GSD, and PPC. In addition, two one-minute aerosol samples were collected on glass sampling filters at 5 and 10 minutes into each run, and they were analyzed spectrophotometrically for drug contents as described below.

Determination of drug contents

The drug contents of aerosolized liposome-encapsulated ciprofloxacin deposited on the sampling filters were determined using a spectrophotometer (UV-160U, Shimadzu Corp., Tokyo. Japan). The glass filters were quartered aseptically, placed in 5 ml of absolute ethanol to disrupt the liposomes and centrifuged at 4,000 RPM for 20 minutes to remove glass fibers. The

ciprofloxacin concentrations in the supernatant were determined at 276 nm and valued extrapolated from a standard curve using know ciprofloxacin standards.

Protection study against respiratory tularemia in mice

For the prophylactic treatment of respiratory tularemia, groups of mice were exposed to aerosols containing liposome-encapsulated ciprofloxacin, free unencapsulated ciprofloxacin or phosphate buffered saline. At eight hours post aerosol exposure, the animals were anesthetized with sodium pentobarbital (50 ml/kg body weight) via the intraperitoneal route. When the animals were unconscious, they were intranasally infected with LD_{50} doses of *Francisella tularensis* which were applied gently with a micropipette into the nostrils. The infected animals were monitored daily for signs of symptoms and for deaths from the infection. At day 14 after infection, the number of mice which survived the lethal bacterial infection was recorded.

Bacterial determination of organ homogenates

To determine the bacterial load in organs of control and treated mice, the lungs, spleens and livers from the mice were aseptically harvested. The organs were then homogenized in 5 ml sterile PBS using a hand-held tissue grinder. The supernatants were then plated for growth in cysteine heart agar plates supplemented with 5% defibrinated rabbit's blood. The inoculated plates were incubated at 37°C for 4 days and the number of colony forming (CFU) of *Francisella tularensis* were determined.

Statistical analysis

The survival rates between the treated and non-treated control groups were compared by the Mann-Whitney unpaired non-parametric one-tailed test (in Stat, Version 1.14; Graph-Pad software, San Diego, California). Differences were considered statistically significant at $P<0.05$.

RESULTS

Size characterizations and measurements of aerosolized liposome-encapsulated ciprofloxacin

The aerosol characteristics of the liposome-encapsulated ciprofloxacin produced by each of the twelve nebulizers are shown in Tables 2 and 3.

Table 2: Nebulizer Characteristics REV determined at a flow-rate of 4 L/min.

Nebulizers	MMAD (μm)	GSD (μm)	PPC (10^6)	Content of Ciprofloxacin deposited on sample filter (μg/mL)
1. DVB7427	1.94	1.66	1.20	5.5
2. A1800	2.62	1.58	2.62	13.1
3. HudTU	2.71	1.47	2.44	10.0
4. Marq	3.10	1.70	2.94	21.7
5. DVB5601	3.25	1.60	3.76	12.3
6. Hosp952	3.26	1.61	3.93	2.3
7. Hosp3753	3.31	1.61	3.50	25.5
8. HudUD2	3.31	1.57	4.46	26.1
9. PurRD	3.36	1.58	4.16	29.8
10. Micro	3.38	1.62	3.90	♥
11. Int	3.46	1.63	4.08	10.9
12. HudMM	♥	♥	4.3	♥

♥ not determined

Table 3: Nebulizer Characteristics REV determined at flow-rate of 6 L/min

Nebulizers	MMAD (μm)	GSD (μm)	PPC (10^6)	Content of Ciprofloxacin deposited on sample filter (μg/mL)
1. HudTU	3.16	1.65	3.37	24.5
2. DVB7427	3.21	1.63	3.41	34.3
3. Marq	3.23	1.84	3.42	12.7
4. PurRD	3.45	1.51	4.36	39.0
5. A1800	3.47	1.58	4.27	27.5
6. Int	3.48	1.62	4.25	33.5
7. Hosp3753	3.49	1.65	4.09	27.0
8. HudMM	3.50	1.53	4.13	40.5
9. Hosp952	3.52	1.59	4.21	34.5
10. DVB5601	3.52	1.58	4.22	27.5
11. Micro	3.74	1.71	3.50	♥
12. HudUD2	3.84	1.57	4.12	30.0

♥ not determined

The aerosol particles for each nebulizer are characterized in accordance to the MMAD, the GSD and the PPC. The MMAD of aerosol particles containing liposome-encapsulated ciprofloxacin generated by the twelve nebulizers ranged from 1.94 to 3.84 μm. The MMAD generated by each nebulizer increased when the air flow was increased from 4 L/min to 6 L/min. The geometric standard deviations of the aerosol particles generated by the twelve nebulizers were small, ranging from 1.47 to 1.70 μm, and were independent of flow-rate.

The PPC of the aerosol particles were determined by the APS at approximately 4 minutes into each run. Referring to Table 2, the PPCs generated by the different nebulizers varied from 1.20 (DVB7427) to 4.46 (HudUD2) million particles. Increasing the airflow from 4 L/min to 6 L/min (Table 3) resulted in the increase in PPCs for nine of the twelve nebulizers.

Drug deposition on sampling filters

The aerosol particles containing liposome-encapsulated ciprofloxacin deposited on the sampling filters at the end of each run were analyzed for ciprofloxacin levels. In comparing the results of the drug content deposited on the sampling filter of the aerosols obtained from each deposition nebulizers at a flow-rate of 6L/min.(see Table 3), the highest drug content was observed from aerosol particles generated with nebulizers HudMM and PurRD (40.5 and 39.0 μg/mL, 0.203 and 0.195 mg/filter). These two nebulizers produced aerosol particles with MMAD 3.5 and 3.45 μm, and PPCs of 4.13 and 4.36 million, respectively. At the same flow rate, the lowest drug deposition was observed with nebulizers Marq and HudTU (12.7 and 24.5 μg/mL) which generated particles with MMAD of less than 3.3 μm and PPCs of less than 3.5 million.

Selection of nebulizers for *in vivo* efficacy study against tularemia infection in mice

Successful therapy of respiratory infection using aerosol inhalation of liposome-encapsulated ciprofloxacin requires the selection of nebulizer(s) which produces aerosol having particles of respirable size and the highest drug deposition. Based on these above criteria, the nebulizers HUdMM and PurRD were considered nebulizers which meet those requirements. The two nebulizers generated aerosol particles of MMAD of about 3.5 μm, and geometric standard deviations of 1.5 μm and yielded drug deposition of about 40 μg/mL. In addition, PurRD was also found to generate higher PPC than HuDMM, and hence it was subsequently selected as the nebulizer of choice for the aerosolization of liposome-encapsulated ciprofloxacin in the efficacy evaluation against *F. tularensis* infection.

Treatment of mice against respiratory tularemia

Turning to Figure 1, the prophylactic efficacy of aerosolized free unencapsulated and liposome-encapsulated ciprofloxacin to protect mice against a respiratory infection against *Francisella tularensis* was evaluated. Mice were pretreated with 10 or 20 minutes exposures to aerosol containing either PBS (control group), free unencapsulated (FC) or liposome-encapsulated ciprofloxacin (LC). At 24 hours post aerosol exposure, the mice were intranasally infected with 10 times 50% lethal doses of *F. tularensis*. The survival rates in these groups of mice at day 14 post infection were compared. Untreated control mice began to succumb to the infection as early as day 5 post infection and by day 13, all mice in the group were dead. Little or no protection was observed in mice treated with aerosolized free unencapsulated ciprofloxacin. All but one of the mice in that group died by day 12 post infection. In mice exposed to 10

minutes of aerosolized liposome-encapsulated ciprofloxacin, the survival rate was significantly higher than the untreated control group (83% versus 0%, $P<0.05$). The highest level of protection was observed in mice exposed to 20 minutes of aerosolized liposome-encapsulated ciprofloxacin (100% vs. 0%, $P<0.01$). These results suggest liposome-encapsulated ciprofloxacin delivered by the aerosol inhalation was highly effective in the prevention of respiratory *F. tularensis* infection in mice.

Referring to Figure 2, the treatment of respiratory infection against *Francisella tularensis* using aerosolized liposome-encapsulated ciprofloxacin and aerosolized unencapsulated ciprofloxacin were compared. Groups of mice were intranasally infected with 10 times the 50% lethal dose of *F. tularensis*. At 24 hours postinfection, the mice were treated with 20 minutes exposures to aerosolized unencapsulated ciprofloxacin or aerosolized liposome-encapsulated ciprofloxacin. The survival rates for these groups of mice at day 14 postinfection are shown in Figure 2. The mice in the untreated control group began to succumb to the infection as early as day 5 postinfection, and by day 9, all mice in the group were dead. Little or no protection was observed in mice treated with aerosolized unencapsulated ciprofloxacin. All the mice in that group died by day 9 postinfection. Among the mice exposed to aerosolized liposome-encapsulated ciprofloxacin, all the mice survived ($P<0.01$ versus the control, unencapsulated ciprofloxacin group). These results suggest that liposome-encapsulated ciprofloxacin delivered by aerosol inhalation is highly effective in the treatment of respiratory *F. tularensis* infection in mice.

Bacteria load of organs from infected and treated mice

The spleens, livers and lungs from the untreated and pretreated mice were isolated at days 7 and 14 post infection, respectively. These organs were homogenized and assayed for the presence of *F. tularensis* growth in cysteine heart agar plates. The results are shown in Table 4.

Table 4: Recovery of *Francisella tularensis* from organs of mice pretreated with aerosolized liposome-encapsulated ciprofloxacin

Group	Organ	CFU
Untreated control♠	Lung	4×10^7
	Spleen	4×10^6
	Liver	3×10^7
Pretreated♣	Lung	2×10^5
	Spleen	0
	Liver	0

♠ CFUs determined at approximately day 7 post infection, before mice were moribound from infection

♣ CFUs were determined at day 14 post infection

The presence of bacteria was only found in the lung at day 14 post infection of mice which were treated with aerosolized liposome-encapsulated ciprofloxacin. In contrast, the lung, spleen, and liver of mice from the control group all had a high amount of bacteria at day 7 post infection. These results suggest that aerosolized liposome-encapsulated ciprofloxacin is potent in the eradication of *F. tularensis* from these tissues.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An aerosol composition comprising a therapeutically effective amount of liposome-encapsulated fluoroquinolone.

2. An aerosol composition as in claim 1, wherein the fluoroquinolone is selected from the group consisting of amifloxacin, cinoxacin, ciprofloxacin, danofloxacin, difloxacin, enoxacin, enrofloxacin, fleroxacin, irloxacin, lomefloxacin, miloxacin, norfloxacin, ofloxacin, pefloxacin, rosoxacin, rufloxacin, sarafloxacin, sparfloxacin, temafloxacin and tosufloxacin.

3. An aerosol composition as claimed in claim 2, wherein the liposome-encapsulated ciprofloxacin is in the form of particles having a mass mean aerodynamic diameter of about 0.5 to 5.0 μm.

4. An aerosol composition as claimed in claim 3, wherein at least about 50% of the particles have a diameter of about 0.5 to 5.0 μm.

5. An aerosol composition as claimed in claim 3, wherein the liposome-encapsulated ciprofloxacin is in the form of particles having mass mean aerodynamic diameter of about 3.45 μm.

6. An aerosol composition as claimed in claim 2, wherein the liposome-encapsulated ciprofloxacin is in the form of particles having a peak particle counts of about 1.2 to 4.4 million.

7. An aerosol composition as claimed in claim 6, wherein the liposome-encapsulated ciprofloxacin is in the form of particles having a peak particle counts of about 4.35 million.

8. A method for treating and preventing respiratory infections using aerosolized liposome-encapsulated fluoroquinolone in a liquid or dry powder form.

9. A method as claimed in claim 8, wherein the liposome-encapsulated fluoroquinolone in aerosol form is administered with a jet nebulizer or a metered dose inhaler.

10. A method for treating and preventing infection by *Francisella tularensis* comprising administering about 5 μg/mL to 40 μg/mL of liposome-encapsulated ciprofloxacin in aerosol form.

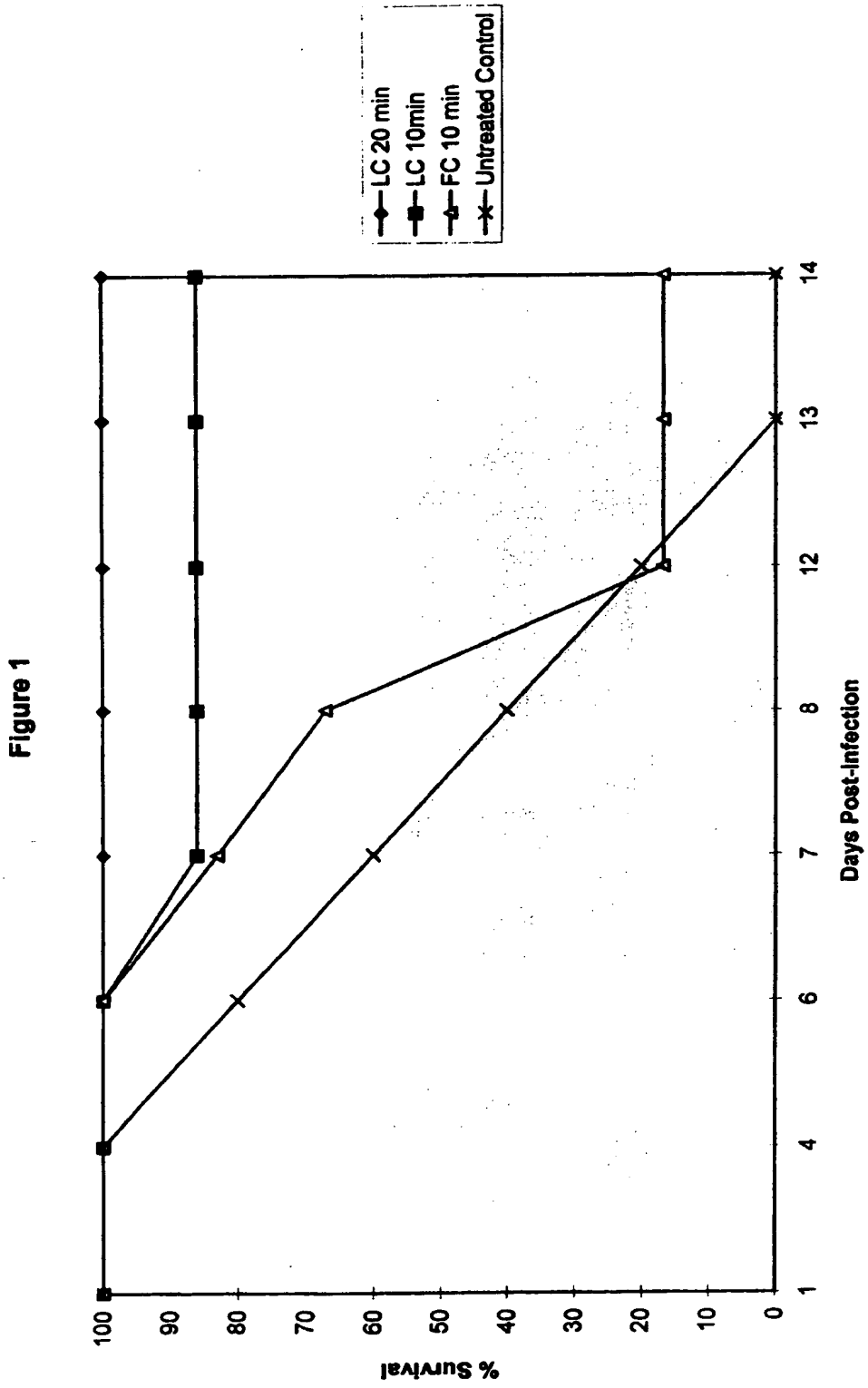
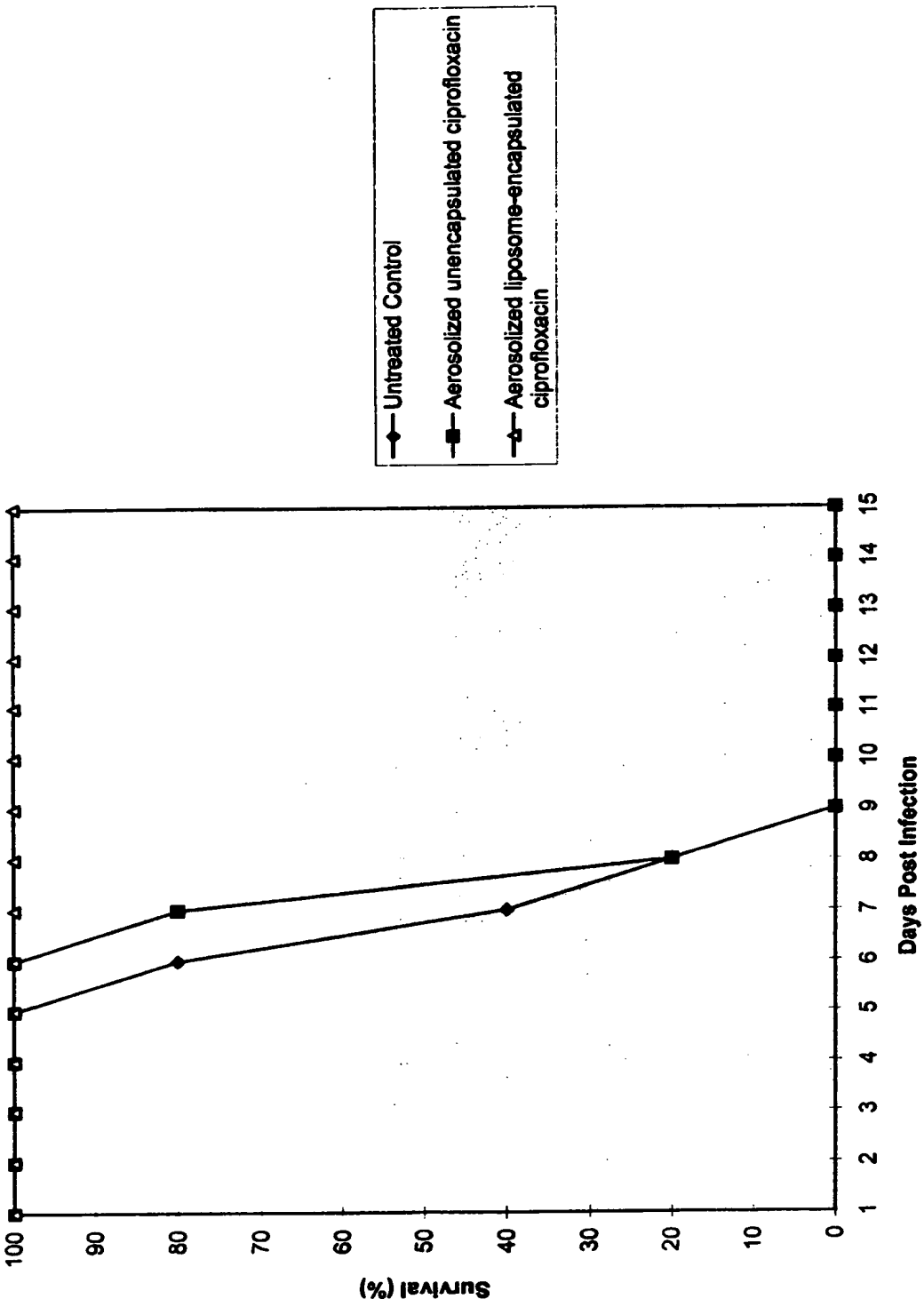

LC 20 min
LC 10min
FC 10 min
Untreated Control
% Survival
100
90
80
70
60
50
40
30
20
10
0
Days Post-Infection
1
4
6
7
8
12
13
14

Figure 1

FIGURE 2


Untreated Control
Aerosolized unencapsulated ciprofloxacin
Aerosolized liposome-encapsulated ciprofloxacin
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
Days Post Infection
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15